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Induction of S-Phase Entry by E2F Transcription Factors Depends on Their Nuclear Localization

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The E2F transcription factors are essential for regulating the correct timing of activation of several genes whose products are implicated in cell proliferation and DNA replication. The E2Fs are targets for negative regulation by the retinoblastoma protein family, which includes pRB, p107, and p130, and they are in a pathway that is frequently found altered in human cancers. There are five members of the E2F family, and they can be divided into two functional subgroups. Whereas, upon overexpression, E2F-1, -2, and -3 induce S phase in quiescent fibroblasts and override G_1 arrests mediated by the $p16^{INK4A}$ tumor suppressor protein or neutralizing antibodies to cyclin D1, E2F-4 and -5 do not. Using E2F-1 and E2F-4 as representatives of the two subgroups, we showed here, by constructing a set of chimeric proteins, that the amino terminus of E2F-1 is sufficient to confer S-phase-inducing potential as well as the ability to efficiently transactivate an E2F-responsive promoter to E2F-4. We found that the E2F-1 amino terminus directs chimeric proteins to the nucleus. Surprisingly, a short nuclear localization signal derived from simian virus 40 large T antigen could perfectly substitute for the presence of the E2F-1 amino terminus in these assays. Thus, nuclearly localized E2F-4, when overexpressed, displayed biological activities similar to those of E2F-1. Furthermore, we showed that nuclear localization of endogenous E2F-4 is cell cycle regulated, with E2F-4 being nuclear in the G_0 and early G_1 phases and mainly cytoplasmic after the pRB family members have become phosphorylated. We propose a novel mechanism for the regulation of E2F-dependent transcription in which E2F-4 regulates transcription only from G_0 until mid- to late G1 phase whereas E2F-1 is active in late G_1 and S phases, until it is inactivated by cyclin A-dependent kinase in late S phase.

E2F was originally defined as a cellular activity required for the transactivation of the adenovirus E2 promoter by the E1A oncoproteins (34). E1A binds directly to pRB, the product of the retinoblastoma susceptibility gene, and to two pRB relatives, p107 and p130 (40). These proteins, often referred to as pocket proteins, are regulators of the E2F family of transcription factors. Five E2F family members have so far been isolated by virtue of their ability to bind directly to pocket proteins and by homology cloning (38). The affinity of the E2Fs toward pocket proteins and DNA is greatly enhanced by their binding to one of two heterodimeric partners, DP-1 and DP-2/3 (38). The DNA tumor virus oncoproteins E1A, human papillomavirus E7, and simian virus 40 (SV40) large T antigen all regulate E2F-dependent transcription by binding and dissociating the pocket proteins from the E2F heterodimers (4).

The E2Fs are believed to regulate the correct timing of transcription of several genes whose products are required for DNA replication (dyhydrofolate reductase, DNA polymerase α, and thymidine kinase) and progression through the cell cycle (cyclin A, cyclin E, CDC2, E2F-1, B-Myb, and p107) (38). Several data suggest that the net effect of the E2F DNA binding site on the transcriptional activity of a promoter can be either positive, like for the dihydrofolate reductase promoter (3), or negative, as in the cases of the B-myb and E2F-1 promoters (15, 17, 22, 33). The structural features determining whether an E2F DNA binding site has a net positive or negative contribution are poorly understood. However, recent results obtained by in vivo footprinting of the CDC2 and B-myb promoters have demonstrated that the E2F sites are occupied only in the G_0 and G_1 phases of the cell cycle, when the E2Fs are bound to pocket proteins (39, 45). Since the pocket proteins are believed to be active repressors of transcription, and since no free E2F is bound to the promoters in G_0 and early G_1, the in vivo footprinting data have led to the proposal of an explanation for why the net effect of the E2F sites in these promoters is inhibitory. Although intriguing, these data do not provide an explanation for the lack of E2F binding to these promoters at later stages of the cell cycle.

Entry into S phase of the cell cycle in normal cells is accompanied by the transcriptional activation of E2F target genes through the phosphorylation of pocket proteins by cyclin-dependent kinases (4). The phosphorylation of pRB by cyclin D/CDK4, cyclin D/CDK6, and cyclinE/CDK2 and of p107 by cyclin D/CDK4, cyclin D/CDK6, and cyclin A/CDK2 results in the release of these pocket proteins from the E2Fs (1, 4). These events are thought to derepress the promoters by releasing the pocket proteins and to unmask the transactivation domains of the E2Fs, which then can act as transcriptional activators. The phosphorylation of pocket proteins occurs late in G_1 and coincides with the restriction point, the moment after which a cell does not depend on serum (growth factors) to complete the ongoing cell cycle (35). In agreement with this definition, CDK4 and CDK6 activities are inducible by serum, and by phosphorylating pRB, these kinases initiate a positive feedback loop that culminates in the activation of cyclin E/CDK2 activity. This feedback mechanism involves autoregulatory transcriptional activation of the E2F-1 promoter, E2F-

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driven transcriptional activation of the cyclin E gene, cyclin E/CDK2-mediated destruction of $p27^{KIP1}$, and further phosphorylation of pRB by cyclin E/CDK2. Once started, this growth-stimulatory mechanism is independent of serum (37). In accordance with this, $Rb^{-/-}$ mouse embryo fibroblasts display diminished serum dependence for their proliferation and contain higher levels of CDK activity (14, 26). Moreover, the upstream regulators of E2F activity, namely pRB, cyclin D, CDK4, CDK6, and $p16^{INK4A}$, are frequently found to be deregulated or mutated in human cancers (40). The importance of E2F-dependent transcription for the initiation of S phase is further emphasized by the findings that overexpression of several members of the E2F family in serum-starved cells results in S-phase entry (18, 27) and that dominant-negative E2F mutants prevent DNA replication (41).

The E2F family members can be divided into two subgroups based on their structure, their affinity toward pocket proteins, and their expression pattern. E2F-1, -2, and -3 bind preferentially to pRB and display very low affinity for p107 and p130 (6, 24). The expression of these E2Fs peaks in late G_1 and S phases (31, 38). E2F-1, -2, and -3 have extended amino termini that contain a cyclin A binding domain (5a, 21, 41a). The members of the other subgroup, E2F-4 and -5, bind to p107 and p130 with high affinity, and recent results have demonstrated that they also bind to pRB (16, 31). E2F-4 and -5 cannot bind cyclin A directly. The expression of E2F-4 is not cell cycle regulated; normal levels of expression are observed even in resting cells (31, 38).

Recently, a functional difference between the two subfamilies was uncovered in our laboratory (27). Whereas E2F-1, -2, and -3 are all capable of inducing S phase in serum-starved fibroblasts, E2F-4 and -5 are not. This phenomenon correlates well with the transactivation potential of the different E2Fs. While E2F-1, -2, and -3 activate a synthetic E2F-responsive promoter very efficiently, E2F-4 and -5 are rather inefficient in this respect. These results suggest that the two E2F subgroups perform distinct functions in the regulation of the mammalian cell cycle and that the transcriptional activities of the two subsets of E2F proteins are regulated in different ways. Alternatively, or in addition to this, our results could be interpreted to mean that the two subgroups of E2Fs target different genes involved in cell proliferation (see below). In order to distinguish between these possibilities, we decided to examine the structural basis for the observed functional differences between the two subsets of the E2F family. We constructed a set of chimeric proteins containing parts of E2F-1 and E2F-4 and tested them for their ability to transactivate an E2F-dependent reporter construct and to induce entry into S phase. The results of these experiments demonstrated that the E2F-1 amino terminus confers both transactivation and S-phase-inducing potentials to E2F-4. Furthermore, we showed that the amino terminus of E2F-1 contains a nuclear localization signal (NLS) that, when linked to these proteins, directs E2F-4 and β-galactosidase (β-gal) to the nucleus. By tagging E2F-4 with a short peptide containing an NLS of SV40 large T antigen, we demonstrated that enforced nuclear localization of E2F-4 is sufficient to explain the gain of transactivation and S-phase-inducing potentials of the E2F-1 amino terminus–E2F-4 chimera. Our data showed that nuclearly localized E2F-4 can activate all the genes necessary for S-phase entry upon overexpression and therefore suggested that the subcellular localization of the E2Fs is regulating their activity. This conclusion is supported by the observation that endogenously expressed E2F-4 protein is nuclear in the early phases of the cell cycle and mainly cytoplasmic after the pocket proteins have become phosphorylated.

Nru I site:

E2F-1: original sequence: GGG GAG AAG ***TCA CGC*** TAT GAG ACC TCA CTG
mutagenic primer: GGG GAG AAG ***TCG CGA*** TAT GAG ACC TCA CTG
amino acids: G_{123} E K S R Y E T S L

E2F-4: original sequence: CCG GGC ACT CCA ***AGC CGG*** CAC GAA AAG AGC CTG
mutagenic primer: CCG GGC ACT CCA ***TCG CGA*** CAC GAA AAG AGC CTG
amino acids: P_{12} G T P S R H E K S L

Psp1406 I site:

E2F-1: original sequence: GAC ATC ACC ***AAC GTC*** CTT GAG GGC ATC CAG
mutagenic primer: GAC ATC ACC ***AAC GTT*** CTT GAG GGC ATC CAG
amino acids: Y_{168} D I T N V L E G I

E2F-4: original sequence: GAC ATT ACC ***AAT GTT*** TTG GAA GGT ATC GGG
mutagenic primer: GAC ATT ACC ***AAC GTT*** TTG GAA GGT ATC GGG
amino acids: Y_{59} D I T N V L E G I

Afl II site:

E2F-1: naturally occuring

E2F-4: original sequence: TAC CAG ATT CAC ***CTG AAG*** AGT GTG AGT GG
mutagenic primer: TAC CAG ATT CAC ***CTT AAG*** AGT GTG AGT GG
amino acids: Y_{179} Q I H L K S V S G

Xba I site:

E2F-1: original sequence: GCG GCC GAC TCG CTC ***CTG GA***G CAT GTG CGG G
mutagenic primer: GCG GCC GAC TCG CTT ***CTA GA***G CAT GTG CGG G
amino acids: A_{379} A D S L L E H V R

E2F-4: original sequence: ATG AGC TCG GAG CTG ***CTG GA***G GAG TTG ATG TCC
mutagenic primer: ATG AGC TCG GAG CTT ***C TA GA***G GAG TTG ATG TCC
amino acids: M_{366} S S E L L E E L M S

FIG. 1. Oligonucleotides used for mutagenesis of E2F-1 and E2F-4 cDNAs. The sequences of the oligonucleotides used to introduce *Nru*I, *Psp*1406I, *Afl*II, and *Xba*I restriction sites into the cDNAs of E2F-1 and E2F-4 are shown. The mutagenesis procedure was silent in terms of amino acid sequence. The bases comprising the introduced restriction sites and the original sequence are shown in boldfaced italic letters. Underlined letters in the mutagenic primer sequences indicate mismatches. The numbers attached to some amino acids as subscripts indicate the positions of these amino acids in E2F-1 or E2F-4. Note that the restriction sites were introduced in regions of amino acid homology.

MATERIALS AND METHODS

Generation of monoclonal antibodies to E2F-4. Female BALB/c mice were immunized subcutaneously with affinity-purified full-length glutathione *S*-transferase–E2F-4 protein, and monoclonal antibodies were prepared by fusing splenocytes to NS-2 mouse myeloma cells 3 days after the final boost as described previously (10). Seven different cell lines producing monoclonal antibodies to E2F-4 were generated by single-cell cloning. The antibodies were designated TFE4x, where x is a number from 1 to 7.

Site-directed mutagenesis. pBSK-containing amino-terminal hemagglutinin (HA)-tagged E2F-1 and E2F-4 have previously been described (13, 27). Site-directed mutagenesis was carried out with the Chameleon double-stranded, site-directed mutagenesis kit (Stratagene) in accordance with the instructions of the manufacturer. The primers that were used for site-directed mutagenesis are shown in Fig. 1.

In vitro translation. In vitro translation was carried out with the TNT coupled reticulocyte lysate system (Promega) in accordance with the instructions provided by the manufacturer.

Cell culture and transfections. C-33A and U-2 osteosarcoma (OS) cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS). For transfections, cells were seeded at a low density and grown to 40% confluence. A DNA-calcium phosphate coprecipitate was pre-

pared according to standard procedures and added to the culture medium. After 12 h, the precipitate was washed away with phosphate-buffered saline (PBS) and fresh, prewarmed medium was added. For transactivation assays, the cells were harvested 24 h after the application of the precipitate. For fluorescence-activated cell sorter (FACS) analysis, immunostaining, immunoprecipitation, and band-shift assays, the cells were harvested 36 h after the application of the precipitate.

Transactivation assays. The following luciferase constructs were used in transactivation assays: pGL3TATAbasic (an E1b TATA box cloned into pGL3 [Promega]) and pGL3TATAbasic-6xE2F (pGL3 containing a TATA box and six E2F binding sites, 5′-TTTCGCGCTTAA-3′). Both plasmids were kindly provided by Ali Fattaey (Onyx Pharmaceuticals, Richmond, Calif.). C-33A cells were grown in 60-mm-diameter dishes and transfected with 1 μg of pGL3TATAbasic-6xE2F, 1 μg of pCMVβ-gal, 1 μg of pCMVneoBam-E2Fx, and 3 μg of sheared calf thymus DNA as described above. The transfected cells were washed free of culture medium with PBS and lysed in situ with 300 μl of luciferase assay lysis buffer (25 mM Tris-phosphate [pH 7.8], 2 mM dithiothreitol, 10% glycerol, 1% Triton X-100). After 5 min, the lysate was transferred to an Eppendorf tube and centrifuged in an Eppendorf tabletop centrifuge at 14,000 rpm for 2 min to spin down cell debris. The clear supernatant was mixed with 20 μl of 10 mM ATP in a luciferase assay tube (Sarstedt), and the luciferase activity was determined in a luminometer (Berthold LB 952 T) with 60 μl of 10 μM luciferin as a substrate. For the determination of the transfection efficiency, the activity of β-gal in the lysate was measured by adding 500 μl of buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM Mg_2SO_4, pH 7.0) and 250 μl of ONPG substrate (4 mg of *o*-nitrophenyl-β-D-galactopyranoside [Sigma Chemical Co.] per ml in 100 mM phosphate buffer, pH 7.0). The lysate was incubated for 30 min at room temperature. The reaction was stopped by adding 200 μl of 1 M sodium carbonate, and the absorbance at 420 nm was read.

FACS analysis. For FACS analysis, C-33A cells were grown in a 60-mm-diameter dish and transfected with 1 μg of pCMVCD20 (43) and 5 μg of pCMVneoBam-E2Fx as described above. The transfected cells were washed twice with PBS to remove the culture medium, and the cells were detached from the dish by incubation with 1 ml of 5 mM EDTA in PBS for 5 min. The cells were transferred to an Eppendorf tube and washed free of EDTA by pelleting them at 3,000 rpm in an Eppendorf microcentrifuge for 2 min and resuspending them in 1 ml of fresh, cold PBS. The cells were pelleted again and resuspended in 20 μl of CD20 staining solution (15 μl of cold PBS plus 5 μl of fluorescein isothiocyanate-labeled anti-CD20 antibody [Becton Dickinson]). After 30 min of incubation on ice with occasional agitation, the unbound anti-CD20 antibody was removed from the cells by two washes with 1 ml of cold PBS. After the second wash, the pelleted cells were carefully resuspended in 100 μl of cold PBS and then fixed by dropwise addition of ice-cold 70% methanol in PBS. The fixation was stopped after 5 min of incubation on ice by pelleting the cells. In order to block hydrophobic sites on the cell surface which get exposed during fixation, the pellet was resuspended in 500 μl of PBS containing 1% FCS. The cells were pelleted again and resuspended in 500 μl of PI buffer (10 mM Tris-HCl [pH 7.0], 5 mM $MgCl_2$, 50 μg of propidium iodide [Sigma] per ml, 100 μg of RNase A per ml). After 60 min of incubation at 37°C, the samples were analyzed with a Becton Dickinson FACScan. The data were analyzed with the CellQuest (June 1994 version; Becton Dickinson) and ModFit LT (Verity) software.

Indirect immunofluorescence of transfected cells. The cells transfected by the calcium phosphate coprecipitation procedure were washed twice with PBS, fixed in cold (−20°C) methanol-acetone (1:1 [vol/vol]) for 5 min, dried, and stored at −20°C. For analysis, the cells were rehydrated and blocked in DMEM plus 10% FCS for 10 min and then incubated with primary (12CA5; undiluted tissue culture supernatant) followed by secondary (Cy3-conjugated goat anti-mouse immunoglobulin G [Jackson Laboratories] diluted 1:100 in DMEM plus 10% FCS) antibodies for 1 h at room temperature with three washes in PBS (5 min each) in between. Excess secondary antibody was washed away (three washes in PBS, 5 min each). The coverslips were then washed once in distilled water and dried. The dried coverslips were mounted on slides with Fluoromount G (Southern Biotechnology Associates, Inc.) and analyzed with a fluorescence microscope (Aristoplan; Leitz). Photographic images were taken on Ektachrome P1600 film (Kodak) with a WILD MPS52 camera (Leica). Alternatively, a JVC KYF55BE three-color digital video camera was used to obtain digitized images, which were then analyzed with the ImageGrabber24 1.2 software (Neotech).

Immunoprecipitation and Western blotting. For immunoprecipitation, the cells were washed free of culture medium and taken off the 100-mm-diameter dish by incubation with 2 ml of 5 mM EDTA in PBS for 5 min. The cells were then pelleted and frozen in liquid nitrogen. The pellets were then lysed by addition of 500 μl of ice-cold IP buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 0.1% Tween 20, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml, 20 U of aprotinin per ml, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM Na_3VO_4) and incubation on ice for 30 min with occasional vigorous vortexing. The samples were then spun at 14,000 rpm in a cooled tabletop centrifuge (Hettich Zentrifugen EBA 12R) for 30 min. The protein concentration in the lysate was determined by the Bradford assay (Bio-Rad), and equal amounts of total protein were incubated in equal volumes of lysis buffer with 20 μl of protein G-Sepharose (Pharmacia) cross-linked to the 12CA5 antibody. Cross-linking of the antibody to protein G was performed as described previously (10). Incubation was continued for 90 min at 4°C on a rotary mixer. The beads were then washed three times in IP buffer, and the bound protein was solubilized by addition of 20 μl of 2× Laemmli sample buffer. Western blotting was performed according to standard procedures with Bio-Rad Minigel apparatuses, semidry blotting equipment (Millipore), and nitrocellulose membranes (Schleicher and Schuell). The following primary antibodies were used: Z83 (anti-p107 and -p130 [25]) [a kind gift of Peter Whyte]), monoclonal antibody G3-245 (anti-pRB; PharMingen), a rabbit polyclonal antibody to cyclin A (Santa Cruz Biotechnology), HE12 (anti-cyclin E; PharMingen), TFD10 (anti-DP-1 [42]), TFE42 (anti-E2F-4 [this report]), 12CA5 (9), D19-2F3-2 (anti-β-gal; Boehringer Mannheim no. 1083 104), KH95, and KH20 (both anti-E2F-1 [13]). Bands were visualized by enhanced chemiluminescence (Amersham).

Bandshift assays. Bandshift assays were performed exactly as described previously (12). The following oligonucleotides were used as probes: E2F wt (5′-ATTTAAGTTTCGCGCCCTTTCTCAA-3′) and E2F mut (5′-ATTTAAGT TTCGATCCCTTTCTCAA-3′).

Cloning of NLS-tagged proteins. To construct an NLS-tagged plasmid, an oligonucleotide encoding the SV40 nuclear localization signal, PKKKRKV (20), was cloned in frame with the HA tag in the plasmid pBSKHA (13), resulting in pBSKHANLS. The cDNAs encoding E2F-4 and the chimera 44441 were cloned in frame with the HA and NLS tags as *Bam*HI-*Eco*RI and *Bam*HI-*Bam*HI fragments, respectively. For expression in mammalian cells, the modified cDNAs were recloned as *Eco*RV fragments into the blunt-ended *Bam*HI site of pCMVNeoBam.

Construction of β-gal fusion proteins. E2F-1(aa 1–126)/β-gal was constructed by replacing an *Nru*I-*Not*I fragment from pBSKHA11111 with an *Eco*RV-*Not*I fragment from pCMVβ-gal (Clontech). E2F-1(aa 120–126)/β-gal was derived from this construct by removing the first 119 amino acids as a *Bam*HI-*Sma*I fragment. The remaining vector was blunt ended and religated. Both cDNAs were recloned as blunt-ended *Eco*RI-*Not*I fragments into the blunt-ended *Bam*HI site of pCMVNeoBam.

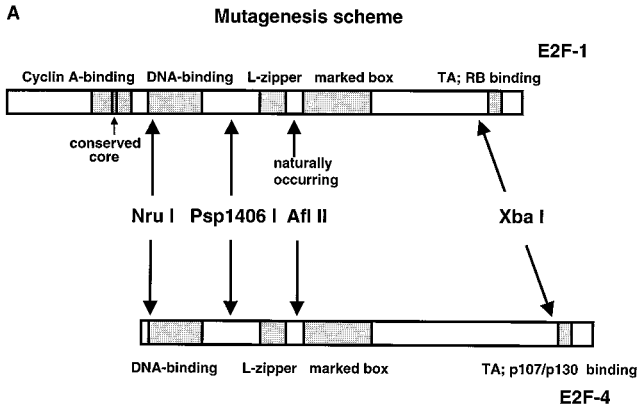
Fractionation and Western blotting. Nuclear and cytoplasmic fractions were prepared by swelling the cells for 10 min in 300 μl of hypotonic buffer A (10 mM Tris [pH 7.5], 10 mM NaCl, 3 mM $MgCl_2$, 1mM EGTA, 10 mM sodium pyrophosphate, 20 μg of aprotinin per ml, 20 μg of leupeptin per ml, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4), subjecting them to 10 slow strokes with a Dounce homogenizer, and spinning them for 5 min at 375 × *g* and 4°C. The supernatant was mixed with an equal volume of 2× Laemmli sample buffer to give the cytoplasmic fraction. The nuclei were washed three times in buffer A plus 0.1% Nonidet P-40 and then lysed in 1× Laemmli sample buffer. Total lysates were prepared by direct lysis of cells in 1× Laemmli sample buffer.

For Western blotting, the following antibodies were used: rabbit polyclonal anti-Eps15 (kindly provided by P. P. DiFiore), rabbit polyclonal anti-Sp1 (Santa Cruz sc-59), rabbit polyclonal antibody to E2F-4 (Santa Cruz sc-866), affinity-purified rabbit polyclonal antibody to E2F-1 (6), rabbit polyclonal anti-p107 (Santa Cruz sc-318), rabbit polyclonal anti-p130 (Santa Cruz sc-317), and monoclonal antibody 245 anti-pRB (PharMingen).

Immunostaining of endogenous E2F-4. Rat1 cells were fixed on glass coverslips in cold (−20°C) methanol-acetone (1:1 [vol/vol]) for 5 min, dried, and stored at −20°C. For analysis, the cells were rehydrated and blocked in DMEM plus 10% FCS for 10 min. The cells were then incubated with TFE44 for 3 h at room temperature. Excess antibody was washed away with PBS (three washes, 5 min each), and biotinylated goat anti-mouse immunoglobulin G secondary antibody (1:100 in DMEM plus 10% FCS; Amersham) was applied for 1 h at room temperature; this was followed by three washes for 5 min each in PBS. The signal was revealed with the Tyramide signal amplification kit from DuPont NEN (TSA-Direct [Red]) by following the instructions of the manufacturer. Briefly, the cells were incubated with horseradish peroxidase-conjugated streptavidin, washed, exposed to tetramethylrhodamine tyramide for 6 min, washed again, and processed for microscopy as described above.

RESULTS

Construction of chimeric E2Fs. To define the protein domain(s) that allows E2F-1, but not E2F-4, to induce S-phase entry, we constructed a set of chimeric E2F cDNAs. As a first step, the cDNAs encoding E2F-1 and E2F-4 were cloned in frame with an amino-terminal HA tag to allow for immunodetection of chimeric proteins with the same antibody. Second, suitable restriction sites were introduced in the cDNAs by site-directed mutagenesis (Fig. 2) (see Materials and Methods). The sites for mutagenesis were chosen to enable the transfer of separate domains from one cDNA to the other without any change in the protein coding properties. To avoid confusion with the wild-type cDNAs, the mutagenized cDNAs encoding E2F-1 and E2F-4 wild-type proteins were named 11111 and 44444, respectively. In this nomenclature, the first digit denotes the amino terminus, the second digit indicates the DNA binding domain, the third digit stands for the dimeriza-



B

crossovers in chimeric E2Fs:				
none	one	two	three	four
11111	11114	11141	11414	14141
44444	11144	11411	14114	41414
	11444	11441	14144	
	14444	14111	14414	
	41111	14411	41141	
	44111	14441	41411	
	44411	41114	41441	
	44441	41144	44141	
		41444		
		44114		

FIG. 2. Construction of chimeric E2Fs. (A) The cDNAs of E2F-1 and E2F-4 were mutagenized to contain compatible restriction sites which enable the exchange of separate protein domains. TA; RB, transactivation-pRB binding domain. (B) A total of 30 chimeric E2Fs were constructed. The following nomenclature was chosen: first digit, amino terminus; second digit, DNA binding domain; third digit, dimerization domain; fourth digit, marked box; and fifth digit, transactivation-pocket protein binding domain. The value of the digit denotes the origin of the respective domain, with 1 meaning derivation from E2F-1 and 4 meaning derivation from E2F-4. See the text for further details.

tion domain, the fourth digit represents the marked box region (24), and the fifth denotes the transactivation-pocket protein binding domain. The values of the digits point out the origins of the respective domains, with 1 referring to E2F-1 and 4 referring to E2F-4.

After demonstrating by in vitro translation that the mutagenized cDNAs encode full-length proteins (data not shown), 30 different chimeric E2F cDNAs were constructed by standard cloning procedures. Together with the two nonchimeric wild-type E2Fs, they represent the 32 possible combinations (Fig. 2B). These cDNAs were then subcloned in the pCMV-NeoBam expression vector.

The E2F chimeras retain their functional integrity. Our knowledge about the domain structure of the E2Fs suggested that several tests could be used to verify the functional integrity of the chimeric proteins, e.g., binding to cyclin A, binding to DNA, binding to and cooperativity with DP-1, binding to pocket proteins, and downregulation of transactivation potential by pocket proteins. For simplicity, for some assays only the results for the single-crossover chimeras (Fig. 2B) are presented.

Figure 3A shows the properties of chimeric E2Fs in terms of binding to cellular proteins. U-2 OS cells were transiently transfected with the different HA-tagged E2F expression plasmids, and Western blotting of immunoprecipitated proteins was performed. These experiments were performed under conditions in which the anti-HA antibody was not limiting, making comparison of the relative amounts of the different E2Fs and of the associated proteins possible. As seen in Fig. 3A, the expression constructs used gave rise to readily detectable levels of protein. Interestingly, all molecules that carry the amino terminus of E2F-1, the carboxy terminus of E2F-4, or both termini appear as multiple bands, most likely due to being in different phosphorylation states (2, 36). In contrast, the molecules that carry the E2F-4 amino terminus and the E2F-1 C terminus appear mainly as one major band. This result suggests that E2F kinases are directed toward their substrate(s) by either the amino terminus of E2F-1 or the carboxy terminus of E2F-4. The E2F-1 amino terminus is known to bind to cyclin A/CDK2, and the E2F-4 C terminus interacts with p107 and p130, both of which can bind to cyclin A/CDK2 and cyclin E/CDK2 (7, 21, 23, 25). Figure 3A also shows that cyclin A and cyclin E associate efficiently with only a subset of chimeras. Cyclin A coimmunoprecipitation can be mediated by both the

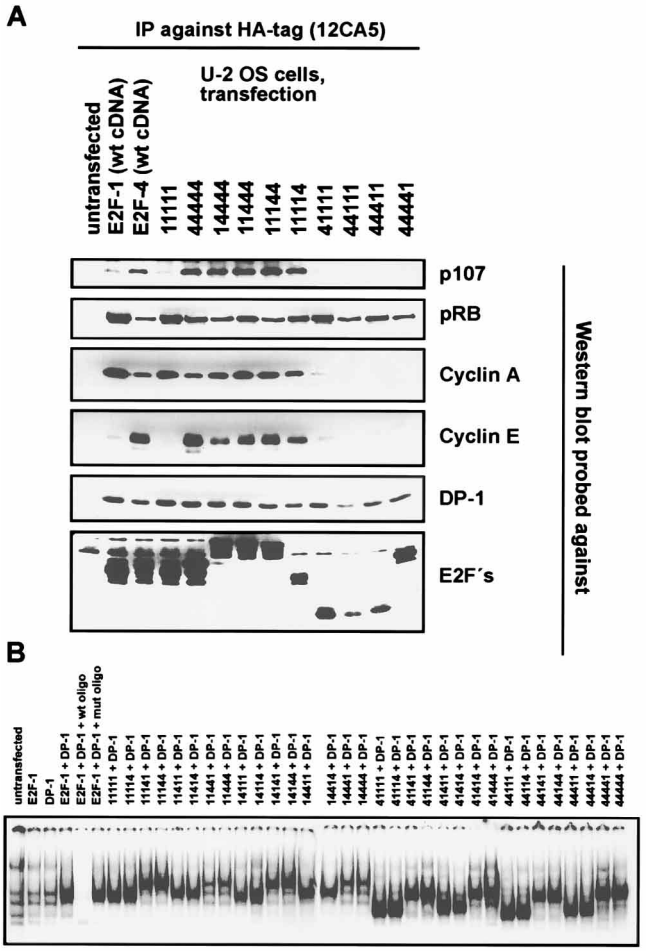


FIG. 3. Biochemical characterization of chimeric E2Fs. (A) E2F-associated proteins. U-2 OS cells were transfected to express the indicated HA-tagged proteins. Thirty-six hours after the application of the precipitate, an immunoprecipitation (IP) was performed with the antibody 12CA5 (directed against the HA tag). Associated proteins were analyzed by Western blotting. (B) E2F gel retardation assay. C-33A cells were transfected to express the indicated proteins. Thirty-six hours after the application of the precipitate, a total cell lysate was prepared and tested for DNA-binding activity by using a ^{32}P-labeled, double-stranded oligonucleotide (oligo) containing an E2F binding site as a probe. The complexes were separated on a nondenaturing 4% polyacrylamide gel with 0.25× Tris-borate-EDTA as the running buffer. wt, wild type; mut, mutant.

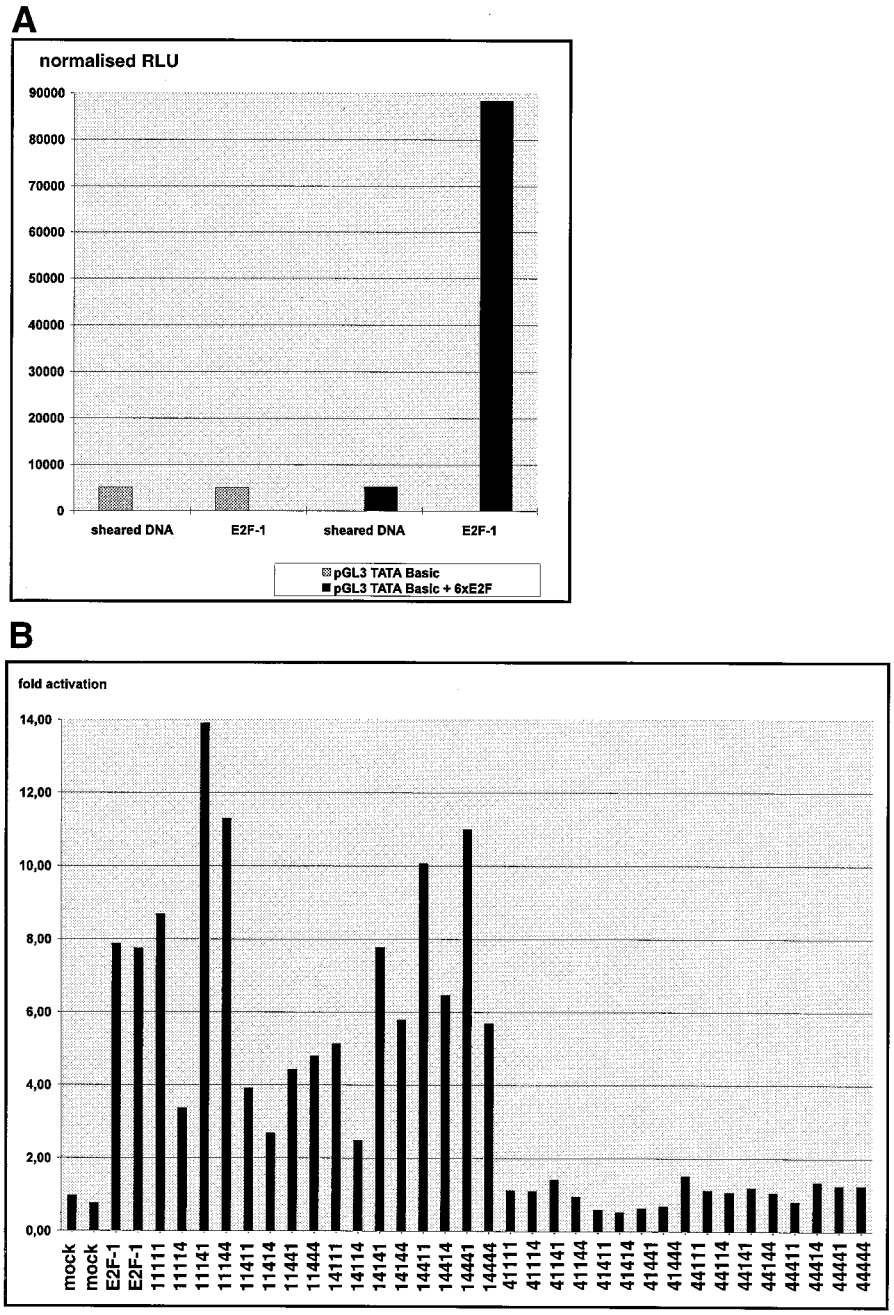


FIG. 4. Transactivation properties of chimeric E2Fs. (A) Transactivation assay. C-33A cells were transfected with either 1 μg of pGL3TATAbasic (the two bars to the left) or 1 μg of pGL3TATAbasic-6xE2F (the two bars to the right) as well as 1 μg of pCMVβ-gal, 3 μg of sheared calf thymus DNA as the carrier, and 1 μg of the indicated plasmids. Thirty-six hours after the application of the calcium phosphate precipitate, luciferase activity was determined and normalized for transfection efficiency, with the activity of β-gal being used as a reference. RLU, relative light units. (B) Transactivation assay. C-33A cells were transfected with 1 μg of pGL3TATAbasic-6xE2F, 1 μg of pCMVβ-gal, 3 μg of sheared calf thymus DNA as the carrier, and 1 μg of the indicated E2F expression plasmids. Twenty-four hours after the application of the calcium phosphate precipitate, luciferase activity was determined and normalized for transfection efficiency, with the activity of β-gal being used as a reference. Experiments done in different cell lines (with either functional or nonfunctional RB) and with various amounts of expression plasmids gave essentially identical results.

amino terminus of E2F-1 and the C terminus of E2F-4, whereas the coimmunoprecipitation of cyclin E appears to be dependent on the presence of the E2F-4 C terminus. The chimeras that display the lowest number of (presumably) differently phosphorylated proteins also coimmunoprecipitate very little cyclin A and cyclin E. Furthermore, Fig. 3A shows that the E2F chimeras bind DP-1 with nearly identical affinities.

The binding of pocket proteins to the different chimeras was also investigated. As demonstrated in Fig. 3A, pRB coimmunoprecipitated with the E2F proteins migrates as a single band corresponding to its hypophosphorylated form. Although in

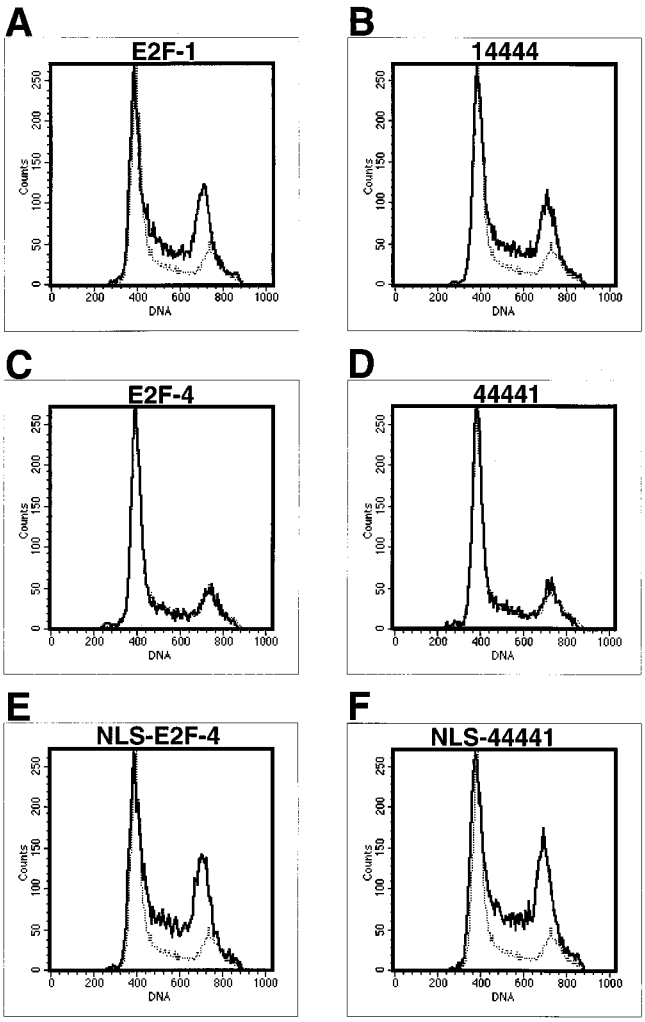


FIG. 5. Induction of S phase by wild-type and chimeric E2Fs. C-33A cells were transfected with 1 μg of pCMVCD20 and 5 μg of the indicated expression plasmids. Thirty hours after the application of the calcium phosphate precipitate, the cells were harvested, and cells expressing the transfected cDNAs were stained with a fluorescein isothiocyanate-labeled antibody directed against CD20. The cells were then fixed in 70% methanol and blocked, and the DNA was stained with propidium iodide. The DNA profiles of 10,000 CD20-positive cells were recorded with a Becton-Dickinson FACScan. DNA profiles of cells expressing E2Fs (thick line) were overlayed on DNA profiles of CD20-expressing cells that received an equal amount of sheared calf thymus DNA instead of an E2F expression construct (dotted fine line). The histograms were normalized vertically according to their G_1 peaks in order to augment visual comparison of the G_1- and S-phase populations. (A) E2F-1; (B) 14444; (C) E2F-4; (D) 44441; (E) NLS-E2F-4; (F) NLS-44441.

this experiment E2F-1 coimmunoprecipitated pRB slightly more efficiently than did E2F-4, we found that the chimeras bound to pRB with comparable affinities, regardless of the origin of their C terminus. In contrast, the binding of p107 to the E2Fs depended on the presence of the C terminus of E2F-4.

The DNA binding properties of the chimeras were investigated as well (Fig. 3B). C-33A cells were transfected with the indicated constructs, and total-cell extracts were tested for their ability to bind a radioactively labeled oligonucleotide containing an E2F consensus binding site. As shown in Fig. 3B, the chimeras gave rise to DNA binding activities similar to those of the wild-type proteins when coexpressed with DP-1. Cooperative DP-1 binding was therefore also conserved in the chimeric proteins. Thus, we conclude that all chimeras retain their expected properties and that all the interchanged domains are fully functional with respect to their previously reported features.

The amino terminus of E2F-1 is required for transactivation and S-phase induction. To gain insight into the contribution of the different protein domains to the observed differences between E2F-1 and E2F-4, the chimeric E2Fs were first tested in a transactivation assay. C-33A cells were transfected with pCMVβ-gal (for the adjustment of transfection efficiency), the different E2F expression constructs, and a reporter construct that directed transcription of the luciferase cDNA from a synthetic promoter containing six E2F consensus binding sites (pGL3TATAbasic-6xE2F). Transactivation of this reporter by the E2Fs is strictly dependent on the presence of the E2F binding sites (Fig. 4A). As shown in Fig. 4B, efficient transactivation could only be detected in chimeras containing the amino terminus of E2F-1. Chimeras with a crossover between the DNA-binding and dimerization domains were slightly less efficient, which was also the case for the chimeric proteins that carry the E2F-4 carboxy terminus that allows for binding to p107 and p130 (C-33A cells express a nonfunctional pRB [36a]). These results demonstrate that the low level of E2F-4-mediated transactivation (in comparison to that mediated by E2F-1) cannot be explained by low efficiency of binding to DNA, DP-1, or pocket proteins.

Next, we asked whether the amino terminus of E2F-1 is sufficient to confer the S-phase-inducing potential of E2F-1 to E2F-4. C-33A cells were transfected with different E2F expression constructs and cotransfected with pCMVCD20 in order to mark transfected cells (43). The cells were stained with anti-CD20 antibody and propidium iodide, and the DNA profiles of CD20-positive cells were analyzed by FACS analysis. Figure 5C shows that E2F-4 was completely unable to cause any shift in the DNA profile of C-33A cells. E2F-1, on the other hand, caused a considerable accumulation of cells in late S phase and a corresponding reduction in the number of G_1 cells (Fig. 5A). Intriguingly, the chimera 14444 induced S phase in transfected C-33A cells almost as efficiently as E2F-1 (Fig. 5B). Moreover, S-phase-inducing activity was observed in all chimeras containing the E2F-1 amino terminus (data not shown). We conclude that the amino terminus of E2F-1 is sufficient to confer the high transactivation potential and the S-phase-inducing properties of E2F-1 to E2F-4.

The amino terminus of E2F-1 acts as an NLS. In our attempts to identify the mechanism that allows the amino terminus of E2F-1 to confer the S-phase-inducing activity of E2F-1 to E2F-4, we investigated a potential NLS in E2F-1 at around amino acid 90. Indeed, immunostaining of E2F-1, overexpressed in U-2 OS cells, showed that E2F-1 is localized in the nucleus (Fig. 6A), whereas a mutant E2F-1 that lacks amino acids 85 to 91 accumulates predominantly in the cytoplasm (Fig. 6B). In contrast, a striking cytoplasmic staining pattern was found for overexpressed E2F-4 (Fig. 6C). However, when the amino terminus of E2F-1 is fused to E2F-4 (mutant 14444), this chimeric protein is transported to the nucleus (Fig. 6D). In fact, nuclear localization was observed in all chimeras that carry the amino terminus of E2F-1 (data not shown), strongly suggesting that the amino terminus of E2F-1 contains an NLS.

Functional impact of an NLS fused to E2F-4. The amino-terminal region of E2F-1 (i.e., the first 126 amino acids) contains, in addition to the putative NLS, a cyclin A binding domain and possibly other unidentified functional regions. In order to investigate the functional importance of the N-terminal NLS in E2F-1, we constructed a plasmid expressing an

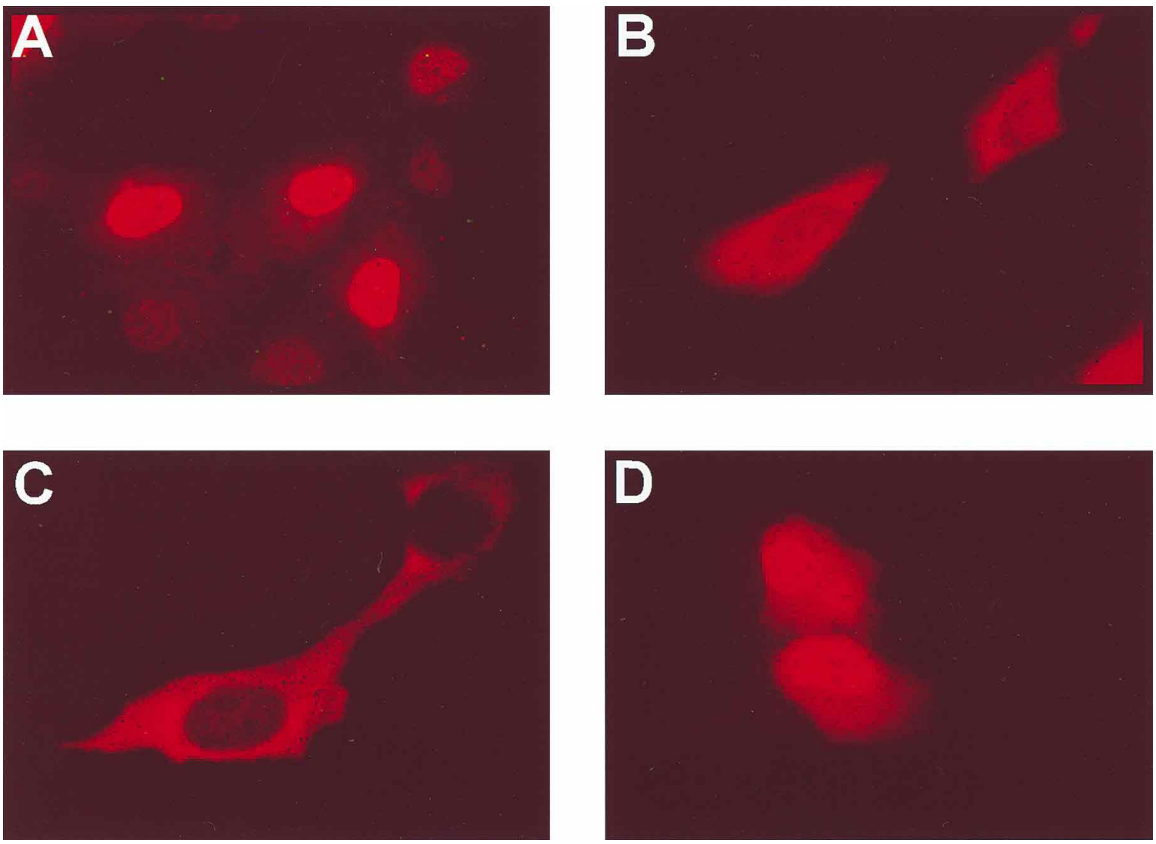


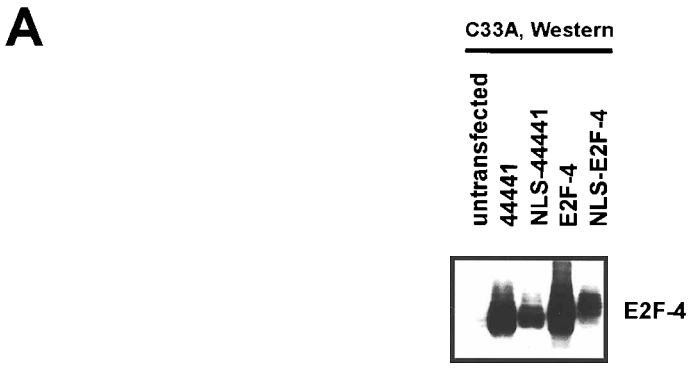
FIG. 6. Subcellular localization of E2Fs. U-2 OS cells were transfected to express the HA-tagged proteins indicated below. Thirty-six hours after the application of the calcium phosphate precipitate, the cells (grown on glass coverslips) were fixed with methanol-acetone (1:1 [vol/vol]). The coverslips were incubated with the antibody 12CA5 (directed against the HA tag) and processed for immunostaining as described in Materials and Methods. (A) E2F-1; (B) E2F-1 dl7 (lacks amino acids 85 to 91); (C) E2F-4; (D) chimera 14444.

E2F-4 protein fused to a short NLS derived from SV40 large T antigen (20). To evaluate a possible role of pocket protein binding, chimera 44441 was also fused to this SV40-derived NLS. Figure 7A shows that these expression constructs produced levels of protein that were easily detectable by Western blotting. Immunostaining revealed that these proteins also are transported to the nucleus, as expected (Fig. 7B).

Surprisingly, when the NLS-tagged proteins were tested for their ability to transactivate pGL3TATAbasic-6xE2F, we found that they were even more efficient than E2F-1 (Fig. 8). NLS-44441 was somewhat more efficient at transactivation than NLS-E2F-4; this could be due to the presence of p107 and p130 and the lack of functional pRB in C-33A. The FACS data shown in Fig. 5E and F demonstrate that both NLS-E2F-4 and NLS-44441 are highly competent at inducing S phase in C-33A cells. The fact that more cells are accumulating in S phase upon expression of NLS-E2F4 and NLS-44441 than in cells expressing the 14444 chimera and E2F-1 is most likely due to the fact that 14444 and E2F-1 still bind to cyclin A via their amino termini. Cyclin A-dependent kinase has previously been shown to downregulate the activity of E2F-DP complexes in late S phase, and this downregulation of E2F-DP activity is required for entry into the G_2 phase of the cell cycle (21). In agreement with this, cells expressing NLS-44441 and NLS-E2F-4 (which cannot bind cyclin A directly) accumulate as a broad peak in late S phase because their activity can't be downregulated by cyclin A-dependent kinase.

Nuclear localization of an E2F-1 amino terminus–β-gal fusion protein. The experiments described above leave open the possibility that sequences downstream of amino acid 126 in E2F-1 cooperate with the amino terminus in the process of nuclear translocation. The amino terminus of E2F-1 is known to bind cyclin A/CDK2 kinase, and phosphorylation of either E2F-1 itself or of an associated DP protein may play a role in the translocation process. In order to investigate whether the amino terminus of E2F-1 is sufficient to serve as an NLS, we constructed fusion proteins containing different pieces of the E2F-1 amino terminus linked to β-gal. These fusion constructs were expressed in U-2 OS cells, and immunostaining with either E2F-1- or β-gal-specific antibodies was performed. As shown in Fig. 9C, E2F-1 accumulates in the nucleus whereas the E2F-1 mutant lacking the first 119 amino acids accumulates mainly in the cytoplasm. The β-gal protein is exclusively cytoplasmic (Fig. 9C). However, when the amino terminus of β-gal is replaced by the first 126 amino-terminal amino acids of E2F-1, the fusion protein is located in the nucleus (Fig. 9C), and this feature is lost again upon removal of the first 119 amino acids from this construct (Fig. 9C). These experiments show that the first 119 amino acids of E2F-1 can direct a heterologous protein to the nucleus and suggest that nuclear translocation of E2F-1 is independent of binding to DP or pocket proteins.

Endogenous E2F-4 is localized in both the nucleus and the cytosol. The results obtained from the overexpression studies



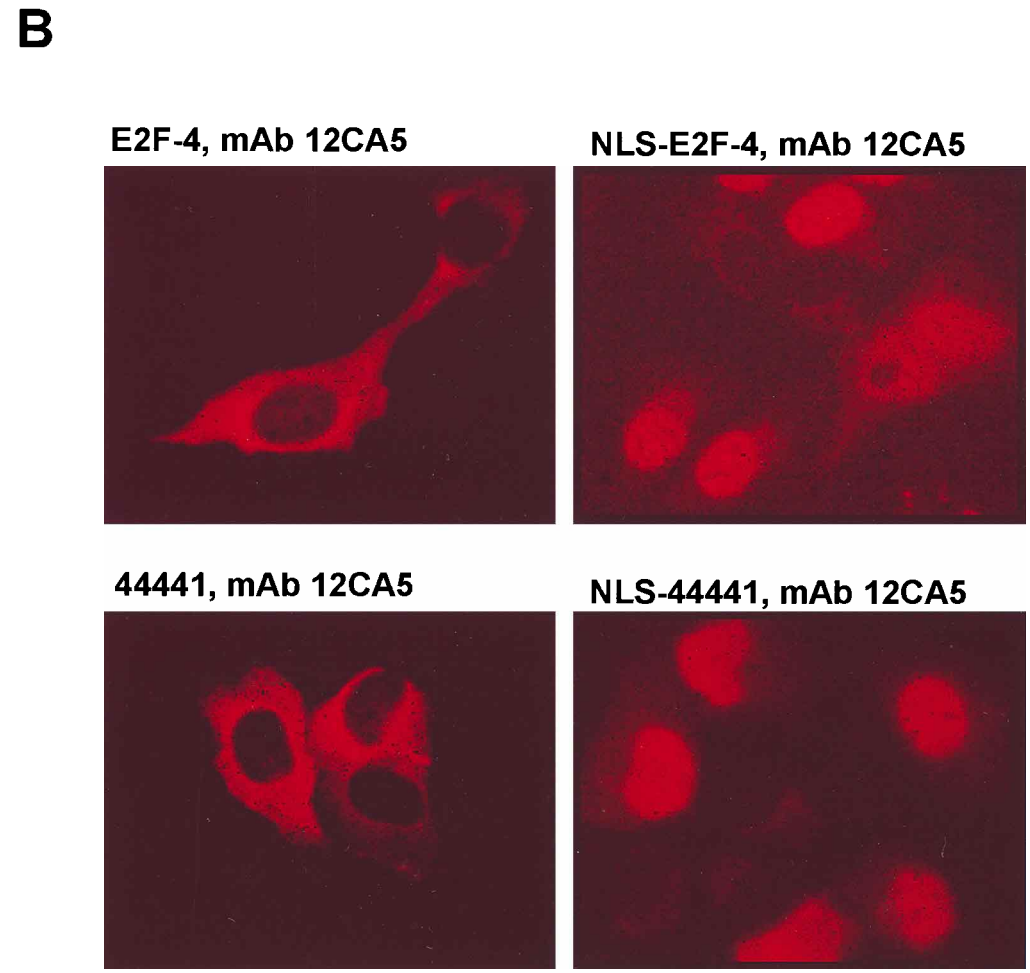


FIG. 7. Immunochemical characterization of NLS-tagged proteins. (A) Western blot. C-33A cells were transfected to express chimera 44441 and E2F-4, both with and without an NLS. Thirty-six hours after the application of the calcium phosphate precipitate, the cells were lysed in Laemmli sample buffer and Western blotting was performed. The blot was probed with TFE42 a monoclonal antibody to E2F-4. (B) Immunostaining. U-2 OS cells were transfected to express chimera 44441 and E2F-4, both with and without an NLS. All proteins were HA tagged. Thirty-six hours after the application of the calcium phosphate precipitate, the cells were processed for immunofluorescence with the 12CA5 antibody as described in Materials and Methods. mAb, monoclonal antibody.

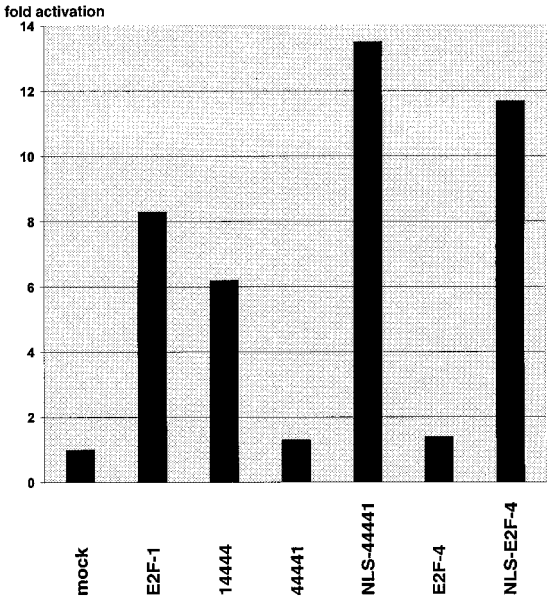


FIG. 8. Functional characterization of NLS-tagged E2Fs by a transactivation assay. C-33A cells were transfected with 1 μg of pGL3TATAbasic-6xE2F-luciferase, 1 μg of pCMVβ-gal, 3 μg of sheared calf thymus DNA as the carrier, and 1 μg of the indicated plasmids. Twenty-four hours after the application of the calcium phosphate precipitate, the luciferase activity was determined and normalized for transfection efficiency, with the activity of β-gal being used as a reference.

with chimeric proteins suggested that the transcriptional activity of E2F-4 is regulated by its subcellular localization. Therefore, we tested whether endogenous E2F-4 is present in the cytoplasm. The distribution of endogenous E2F-4 was evaluated in total-cell, nuclear, and cytosolic extracts prepared from a panel of human and mouse cell lines. As shown in Fig. 10A, between 70 and 90% of the total amount of E2F-4 is found in the cytosol in asynchronously proliferating cells.

The abundance of E2F-4 in the nucleus fluctuates in a cell cycle-dependent manner. Since E2F-4, being a transcription factor, is assumed to exert its main function in the nucleus, we were wondering if cell cycle-regulated fluctuations in the concentration of the nuclear pool of endogenous E2F-4 can be observed. Rat1 fibroblasts were made quiescent by serum starvation, and the cells were harvested for total-cell, nuclear, or cytosolic extract preparations, with 3-h intervals after serum addition until a whole cell cycle was completed (Fig. 10B). The extracts were evaluated for the presence of E2F-4, E2F-1, pRB, p107, p130, Eps15, and Sp-1 by Western blotting. The last two proteins served as controls for the quality of separation of the nuclear and cytosolic fractions in the extracts; Eps15 has previously been described as a cytosolic protein (8), and Sp1 has been found to be a nuclear transcription factor (19). As seen in Fig. 10C, E2F-4 is present in total-cell extracts throughout the cell cycle, with a slightly increased abundance after serum stimulation. E2F-4 is also present in the cytosol throughout the cell cycle, and, importantly, it is less abundant in the cytosol in quiescent cells and just after serum stimulation. Strikingly, the abundance of E2F-4 in the nucleus fluctuates throughout the cell cycle, with a substantial part of total E2F-4 in the nucleus in quiescent cells and for the first 6 to 9 h after serum stimulation. In late G_1 and S phases, the level of endogenous E2F-4 in the nucleus decreases, and it increases again when a substantial number of cells have entered the next G_1 phase. Interestingly, p130 and pRB are phosphorylated just prior to the disappearance of E2F-4 from the nucleus (Fig. 10C). The phosphorylated forms of pRB have previously been shown to be loosely associated with nuclear structures (30), and in agreement with those results, we found phosphorylated pRB in the cytosolic fraction of the cells. p107 and p130 behaved similarly to pRB, with phosphorylated forms being observed in the cytoplasmic fraction. At the time of disappearance of E2F-4 from the nucleus and just after the phosphorylation of p130 and pRB, E2F-1 is synthesized (Fig. 10C), and it stays primarily in the nucleus during the remainder of the cell cycle. In agreement with a recent report (44), we observed an increased abundance of p107 at the G_1-S boundary. Similar results for the subcellular distribution of E2F-1 and E2F-4 during the cell cycle were observed in NIH 3T3 cells (data not shown).

Because of the intrinsic technical problems inherent in fractionation of cells (the fractions are seldom 100% pure), we wanted to confirm our results on the subcellular localization of endogenous E2F-4 by another method. To do this, we tested several monoclonal and polyclonal antibodies specific for E2F-4 for their ability to recognize E2F-4 in an indirect immunofluorescence assay. Although it can be difficult to interpret the specificity of the staining pattern in indirect immunofluorescence, two monoclonal antibodies (TFE42 and TFE44) recognizing different epitopes in E2F-4 gave similar staining patterns that were consistent with the cell fractionation data. The strongest signals were obtained with the TFE44 antibody, and this antibody was used to determine the subcellular localization of E2F-4 in synchronized Rat1 fibroblasts (Fig. 11). In agreement with the subcellular fractionations, this method showed that E2F-4 is mainly nuclear in quiescent cells and just after serum stimulation (Fig. 11A and B) and that it is nuclear and cytosolic in late G_1 phase (Fig. 11C and D). Interestingly, we found that E2F-4 is primarily cytosolic during the remainder of the cell cycle (Fig. 11E to G), while it becomes nuclear again when cells enter the next cell cycle (Fig. 11H and I).

DISCUSSION

There is considerable evidence that the E2F transcription factors play a major role in the regulation of transcriptional events that lead to entry into S phase and, therefore, a new cell cycle. In agreement with this, overexpression of E2F-1, -2, and -3 can induce S-phase entry in resting cells; in contrast, E2F-4 and -5, although closely related to E2F-1, -2, and -3, are incapable of doing so (27). Using chimeric E2Fs, we showed here that the E2F-1 amino terminus is sufficient to confer S-phase-inducing potential to E2F-4. We also showed that the E2F-1 amino terminus directs the chimeric proteins to the nucleus. Nuclear localization and S-phase-inducing potential were strongly correlated with high activity of the chimeras as transactivators of transcription. When E2F-4 was tagged with an NLS, it behaved similarly to chimera 14444 (E2F-4 carrying the E2F-1 amino terminus). Taken together, our results strongly suggest that the different biological activities of E2F-1 and E2F-4 can be ascribed to structural differences in their amino termini.

While these experiments were in progress, Mann and Jones (29) demonstrated that when fused to the carboxy terminus of E2F-4 the amino-terminal 284 amino acids of E2F-1, containing the cyclin A, DNA, and DP-1 binding domains of E2F-1, were able to induce S-phase entry in quiescent cells and to overcome a p16-mediated cell cycle block. Based on these results, the authors suggested that the differences in the abilities of the E2Fs to induce S phase most likely were due to the

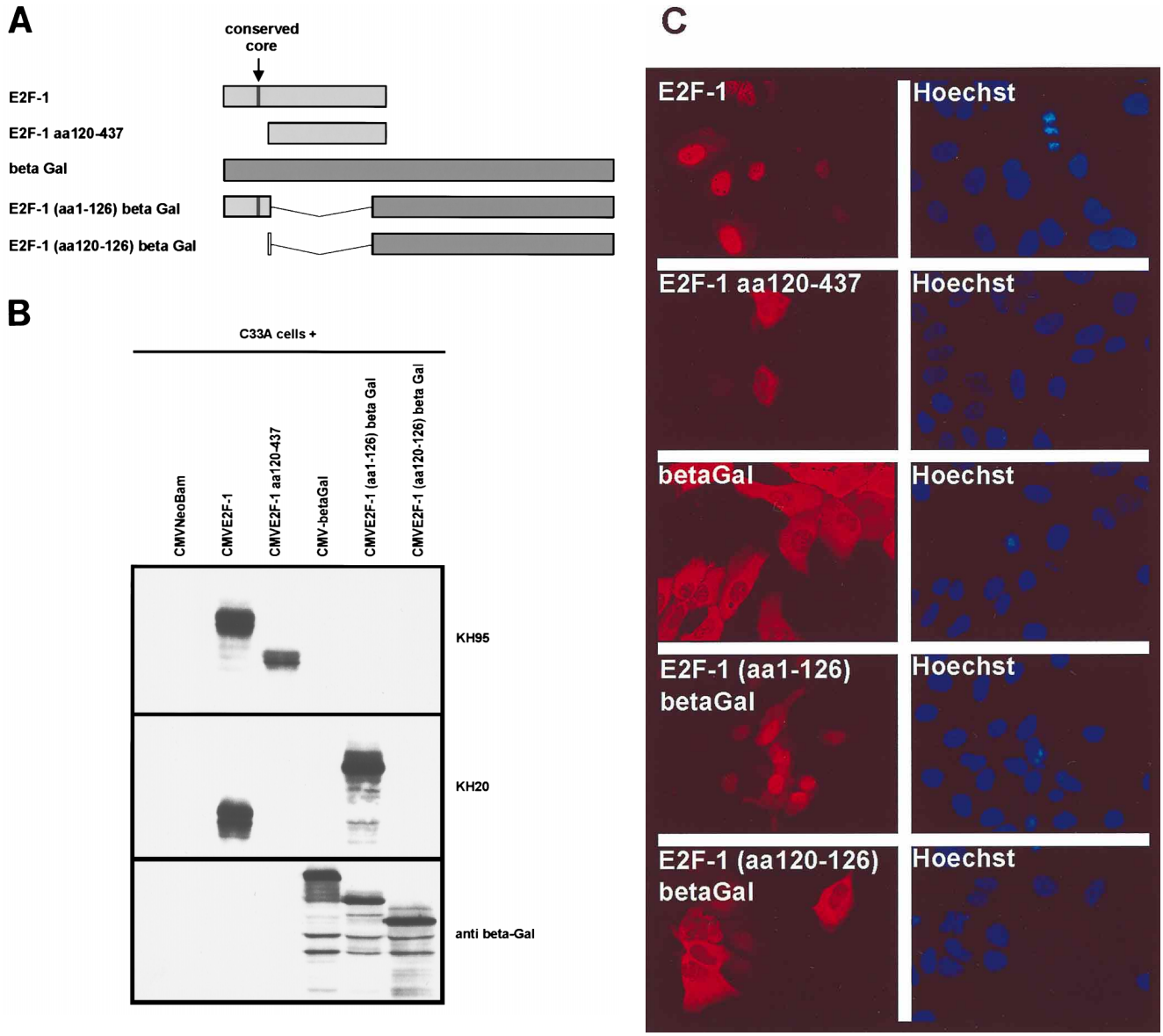


FIG. 9. Subcellular localization of E2F-1–β-gal chimeric proteins. (A) Schematic representation of the E2F-1–β-gal chimeras. (B) Western blot. The molecules depicted in panel A were expressed in C-33A cells and analyzed by Western blotting with different antibodies. KH95 recognizes a C-terminal epitope in E2F-1 and reveals E2F-1 wt and E2F-1(aa 120–437). KH20 binds an N-terminal epitope in E2F-1 and recognizes E2F-1 wt and E2F-1(aa 1–126)/β-gal. The monoclonal antibody D19-2F3-2 binds to β-gal (downstream of amino acid 399) and reveals β-gal, E2F-1(aa 1–126)/β-gal, and E2F-1(aa 120–126)/β-gal. (C) Immunofluorescence. U-2 OS cells were transfected with the constructs shown in panel A and stained with either the monoclonal antibody KH95 [in the case of E2F-1 and E2F-1(aa 120–437)] or the monoclonal antibody D19-2F3-2 against β-gal [in the case of β-gal, E2F-1(aa 1–126)/β-gal, and E2F-1(aa 120–126)/β-gal]. The nuclei were counterstained with Hoechst 33258.

DNA binding portion of these molecules. By performing a more detailed analysis, we showed in this study that the first 126 amino acids of E2F-1, which lie upstream of the DNA and DP-1 binding domains, are sufficient to confer S-phase-inducing and transactivation potentials to E2F-4. Furthermore, within the resolution of the assays used, we ruled out the possibility that any other combination of protein domains derived from E2F-1 and E2F-4 contributes to this effect. This result strongly suggests that the E2F-1 amino terminus serves a previously unidentified function. We have provided evidence that this function is to direct E2F-1 to the nucleus.

Mutational analysis of the E2F-1 amino terminus resulted in the identification of a short stretch of amino acids at around amino acid 90 whose presence is required for E2F-1 to be transported to the nucleus. This stretch of sequence is identical to the conserved core of the cyclin A binding domain identified by others (21), and deletion of this region has been shown to dramatically reduce the ability of E2F-1 to bind cyclin A. We cannot formally exclude the possibility that binding to cyclin A is a prerequisite for nuclear transportation of E2F-1. We showed, however, by linking the E2F-1 amino terminus to a fragment of β-gal that phosphorylation of a DP molecule does not seem to be important since the E2F-1 amino terminus–β-gal chimera (which cannot bind DP) is transported to the

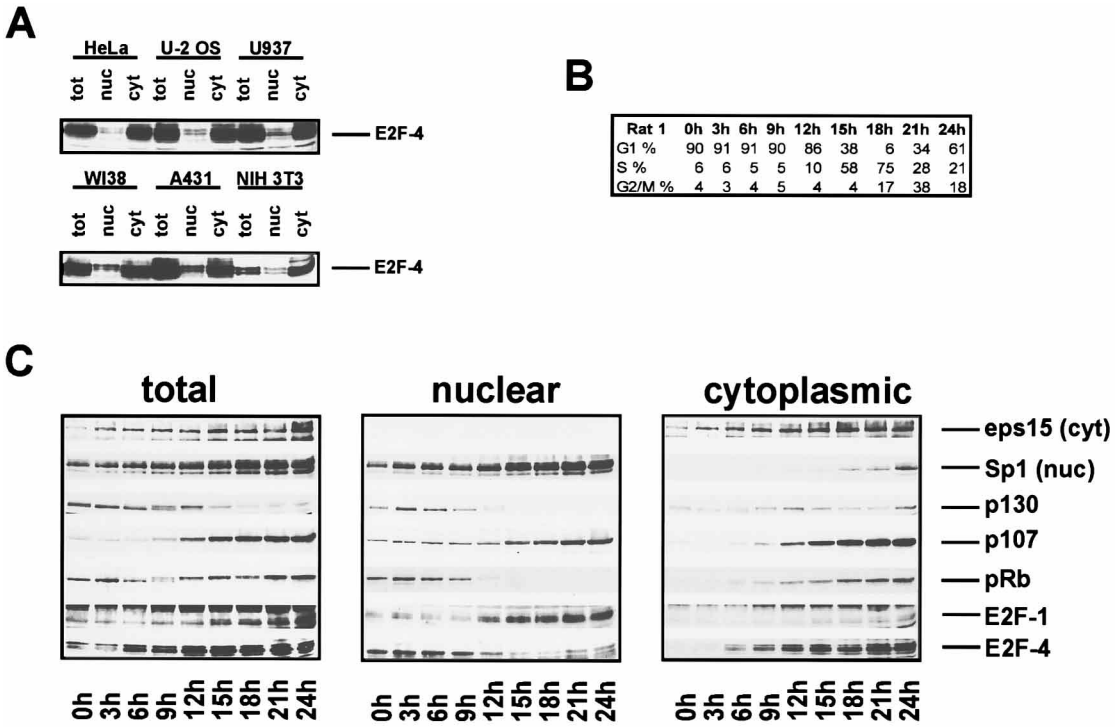


FIG. 10. Cell cycle-regulated nuclear localization of E2F-4. (A) Endogenous E2F-4 accumulates mainly in the cytoplasm in asynchronously proliferating cells. Cytoplasmic (cyt) and nuclear (nuc) fractions of the indicated cell lines were analyzed in parallel with total lysates (tot) for their contents of E2F-4 protein by Western blotting. (B) Rat1 cells were rendered quiescent by serum starvation. After readdition of serum, samples were harvested every 3 h and analyzed by FACS. The cell cycle distribution was estimated with the ModFit program. (C) Cytoplasmic and nuclear extracts were prepared from Rat1 cells in parallel and analyzed, together with total lysates, for their contents of the indicated cell cycle regulators by Western blotting. Eps15 served as a marker for the purity of the cytoplasmic fraction, whereas Sp-1 was used for monitoring the purity of the nuclear fraction.

nucleus. This point is further corroborated by the observation that virtually all of the overexpressed E2F-1 is located in the nucleus, even at expression levels that exceed the level of endogenous DP and cyclin A proteins.

We showed that 70 to 90% of endogenous E2F-4 is in the cytoplasm in asynchronously proliferating cells. Furthermore, we found that the nuclear localization of E2F-4 is cell cycle regulated and that E2F-4 is nuclear from G_0 until mid-G_1 phase and mainly cytoplasmic in late G_1, S, and G_2 phases. In contrast, endogenous E2F-1 is absent from resting cells and is predominantly nuclear in late G_1 and S. Thus, the subcellular localization of the overexpressed E2F-1 and E2F-4 proteins reflects the localization of endogenous E2F-1 and E2F-4. Furthermore, the subcellular localization of the E2Fs also offers an explanation for their different effects on cell cycle progression when they are overexpressed.

The demonstration that E2F-4, when overexpressed, can promote S-phase progression when forced into the nucleus by the presence of an NLS suggests that E2F-4, like E2F-1, can bind to and activate transcription of all the genes necessary for S-phase progression. Taken together with the demonstration that E2F-4 is predominantly nuclear in the early phases of the cell cycle and cytosolic from late G_1 until G_2/M, our data may suggest a cascade of regulatory events in which E2F-4 regulates S-phase-related genes in early phases of the cell cycle and E2F-1 regulates a very similar set of genes in late G_1 and S phases. We speculate that E2F-4 downregulates the transcription of E2F target genes in G_0 and early G_1 by recruiting to the respective promoters pocket proteins, which act as transcriptional repressors. Phosphorylation of the pocket proteins would lead to derepression of these promoters, including the E2F-1 promoter. Since free E2F-4 has a shorter half-life than E2F-4 bound by pocket proteins (11), the nuclear concentration of E2F-4 would drop in late G_1 phase. E2F-1 is expressed after the release of phosphorylated pocket proteins from its promoter, and it increases further the expression of E2F target genes until its activity is downregulated by cyclin A/CDK2 in late S phase. This model also accounts for the lack of functional differences in chimeras carrying either the E2F-1- or the E2F-4-derived DNA binding domain. The results of recent in vivo footprinting experiments using the CDC2 and B-myb promoters are in conflict with such a model, however, since these experiments showed that the E2F DNA binding sites of these promoters are occupied only in G_1 (39, 45). Thus, it appears that E2F-1, -2, and -3 are prevented from binding to the E2F sites of these promoters by some unknown mechanism(s) that could include chromatin structure and/or possible interactions with other proteins binding to the CDE/CHR elements of these promoters (46). Therefore, further experiments will be required in order to reveal the mechanism(s) that determines the accessibility of a promoter to regulation by a member of the E2F family.

How is E2F-4 translocated into the nucleus? One way of addressing this question is to coexpress the E2Fs with their various known binding partners. By coexpressing E2F-1 or E2F-4 together with DP-1 in U-2 OS cells and then immunostaining, we demonstrated that DP-1 is localized in the nucleus when coexpressed with E2F-1 and in the cytoplasm when coexpressed with E2F-4 (32). Therefore, it seems that the subcellular localization of the E2F–DP-1 complexes is decided by

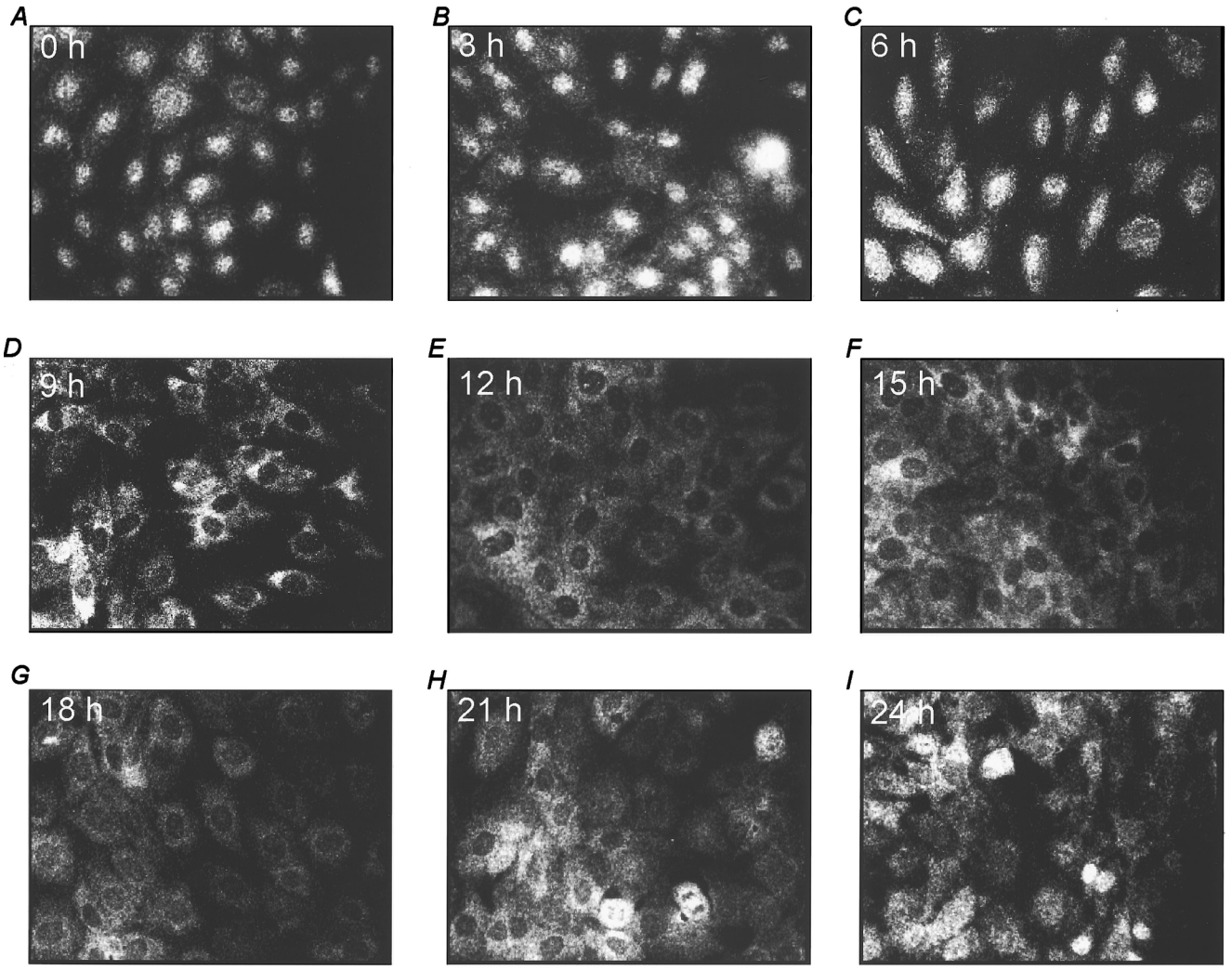


FIG. 11. Immunofluorescence of endogenous E2F-4 and E2F-5. Rat 1 cells were synchronized by serum starvation, fixed in cold (−20°C) methanol-acetone (1:1 [vol/vol]) at the indicated time points after readdition of serum, and stained for endogenous E2F-4 with the monoclonal antibody TFE44. The signal was revealed with the Tyramide signal amplification kit [TSA-Direct (Red)] from DuPont NEN by following the instructions of the manufacturer (see Materials and Methods for details). (A and B) E2F-4 is nuclear is quiescent cells and in early G_1. (C and D) E2F-4 starts to accumulate in the cytoplasm in late G_1, with a considerable fraction still being nuclear. (E to G) E2F-4 is mainly cytoplasmic in the S and G_2 phases of the cell cycle. (H and I) E2F-4 reenters the nucleus in the next G_1 phase. Longer exposure times have been used in panels A to C to compensate for the lower abundance of E2F-4 in that part of the cell cycle.

the E2F partner. Interestingly, similar experiments with E2F-1, E2F-4, and DP-2/3 showed that DP-2/3 can direct nuclear translocation of E2F-4 (5, 28). Since DP-2/3, in contrast to DP-1, contains an NLS, this result is not extremely surprising. We are currently trying to extend these data to the endogenously expressed E2F-4 by performing experiments to determine whether the endogenous cytoplasmic and nuclear E2F-4 molecules bind preferentially to DP-1 and DP-2/3, respectively.

In this paper, we have focused on E2F-1 and E2F-4 as representatives of the two subfamilies of E2F transcription factors. Based on the expression pattern, the subcellular localization upon overexpression, and the structures of the molecules, we speculate that E2F-2 and -3 behave similarly to E2F-1 and that E2F-5 acts similarly to E2F-4. Future studies are required to justify these speculations.

ACKNOWLEDGMENTS

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